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Epigenetic demethylation of sFRPs, with emphasis on sFRP4 activation, leading to Wnt signalling suppression and histone modifications in breast, prostate, and ovary cancer stem cells

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ABSTRACT

The expression and levels of secreted frizzled-related proteins (sFRPs), important Wnt signalling antagonists, have been reported to be reduced in various cancers, and are associated with disease progression and poor prognosis. During tumour development, all sFRP (1, 2, 3, 4, and 5) genes are hypermethylated, causing transcriptional silencing. sFRPs have an ability to sensitize tumour cells to chemotherapeutic drugs, enhancing cell death. Reduced Wnt signalling is associated with loss of cancer stem cell (CSC) viability. We investigated the possible involvement of methylation-mediated silencing of the sFRP gene family in CSCs derived from breast, prostate, and ovarian tumour cell lines. Real-time RT-PCR studies indicated that loss or downregulation of sFRP (1-5) expression in tumours is associated with promoter hypermethylation. Additionally, CSCs derived from all tumour cell lines with sFRP (1-5) promotor hypermethylation expressed sFRP (1-5) mRNA after treatment with 5-Azacytidine (5-Aza), especially sFRP4, implying that DNA methylation is the predominant epigenetic mechanism for sFRP (1-5) silencing. Furthermore, post-translational modification (PTM) in total and histone proteins was observed post 5-Aza and sFRP4 treatment. Protein levels of Wnt downstream signalling components (GSK3β, active β-catenin, and phospho β-catenin) and epigenetic factors of histones (acetyl histone H3, and H3K27me3) affecting PTM were analysed.

Our findings suggest that downregulation of sFRP4 expression in endocrine-related cancers can be attributed to aberrant promoter hypermethylation in conjugation with histone modification, and indicate the important role of methylation-induced gene silencing of sFRP4 in survival and proliferation of CSCs derived from these cancers.

1. Introduction

Various factors contribute to the progression of cancer and benefit the alterations of tumour cells. These alterations include cell cycle deregulation, resistance to apoptosis, and aberrant activation of signalling pathways that promote invasiveness, treatment-resistance, and angiogenesis (Nusse and Clevers, 2017). The cellular epigenetic alterations are due to the fundamental changes within the tumour cell epigenome; hence, studying epigenetic modulations in tumour cells could explain their role in the initiation and progression of human cancer (Chik et al., 2011; Pogribny et al., 2012; Timp and Feinberg, 2013). Mutated epigenetic modulators in cancer are the most common cause leading to oncogenic cellular reprogramming and promoting uncontrolled self-renewal capacity (Wainwright and Scaffidi, 2017). Furthermore, epigenetic modulations such as DNA methylation, histone modifications, and chromatin remodelling result in promoting malignant phenotypes at various stages of cancer. Aberrant epigenetic alterations may transform progenitor cells to different lineages, leading to tissue differentiation (Huang and Esteller, 2010). More importantly, epigenetic alterations can result in variability in treatment response. It has been demonstrated that a small subpopulation of tumour cells is resistant to chemotherapeutic drugs (Deshmukh et al., 2017b) in a variety of cancers such as breast, ovary, and prostate, which could be due to the aberrant expression of key epigenetic factors (Toh et al., 2017). This sub-population of tumour cells is referred to as cancer stem cells (CSCs), and they possess stem-like properties with differentiation capacities and

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high regenerative characteristics (Schatton et al., 2008). The emergence of CSCs requires elaborate epigenome reorganisation, which facilitates the integration of epigenetic mechanisms to establish intra-tumoural heterogeneity (Wainwright and Scaffidi, 2017).

The modulation of epigenetic factors allows targeting downstream signalling pathways, which determine the CSCs' state. The Wnt signalling pathway plays a crucial role in CSC proliferation, differentiation, and self-renewal (Pohl et al., 2017; Lamb et al., 2013). Loss of negative regulators such as glycogen synthase kinase 3β (GSK3β), and accumulation of β-catenin in the cytoplasm leads to upregulation of gene transcription and enhances tumour development (Bafico et al., 2004; Bilic et al., 2007; Gordon and Nusse, 2006; Veeck et al., 2006). Aberrant activation of Wnt signalling inactivates GSK3β, thus enabling increased accumulation of unphosphorylated β-catenin. Accumulated cytoplasmic β-catenin translocates to the nucleus, activating target genes responsible for tumour development and progression (Clevers, 2006; Clevers and Nusse, 2012; Nusse and Clevers, 2017). Therefore, Wnt antagonists such as the secreted frizzled-related proteins (sFRPs 1-5) play a crucial role in controlling pathway aberrations and thus tumour development (Rattner et al., 1997). SFRP4 has the ability to chemo sensitize CSCs from various tumours, reducing their viability (Deshmukh et al., 2017b; Warrier et al., 2013, 2014).

The *sFRPs* are shown to be hypermethylated in various tumours, inducing transcriptional silencing (Schiefer et al., 2014; Suzuki et al., 2002). These epigenetic modification of *sFRPs* (Schiefer et al., 2014; Suzuki et al., 2004) indicate DNA methylation as an important mechanism to target gene silencing (Siegfried and Simon, 2010). Furthermore, histone modifications such as acetylation, methylation, and phosphorylation can alter gene expression. These alterations influence biological processes such as DNA damage repair and uncoiling of chromatins (Bannister and Kouzarides, 2011; Deshmukh et al., 2017a; Kouzarides, 2007). Histone modifications and their co-factors are crucial to understand, such as post-translational modification (PTM) that is responsible for accelerating transcription and hindering DNA methylation (Eberharter et al., 2005; Kim et al., 2009). Thus, histone modifications are involved in various epigenetic processes of tumour development and progression (Zentner and Henikoff, 2013).

The objective of this study was to investigate the involvement of methylation-mediated silencing of the *sFRP* gene family. Our lab has been investigating sFRP4 since its first discovery (Wolf et al., 1997) and has extensively published research on the role of sFRP4 in various hallmarks of cancer (Deshmukh et al., 2017b; Drake et al., 2009; Fox et al., 2013; Longman et al., 2012; Maganga et al., 2008; Perumal et al., 2017, 2016; Pohl et al., 2015; Saran et al., 2012, 2017; Visweswaran et al., 2015). Hence, we have specifically examined the effect of sFRP4 on histone modifications in CSCs derived from breast, prostate, and ovarian tumour cell lines. To study the hypermethylation/demethylation of DNA, the DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) inhibitor 5-Azacytidine (5-Aza) was used, and CSCs were treated with sFRP4 to examine the PTM effect on histone modification and its co-factors.

2. Results

2.1. Increasing 5-Azacytidine concentration reduces CSC viability

Using a CCK-8 viability assay, it was observed that a high dose (60 μM) of 5-Aza at 48 h significantly inhibits the viability of CSCs compared to a lower dose (30 μM). The viability inhibition between 30 μM and 45 μM 5-Aza treated CSCs was significantly less compared to the higher dose of 60 μM 5-Aza (Fig. 1). Similar patterns were observed in CSCs derived from all cell lines. Therefore, to produce less cytotoxic stress on CSCs, while maintaining an equivalent inhibition potency, we decided to select a lower dose of 30 μM 5-Aza for CSC treatment in subsequent experiments.

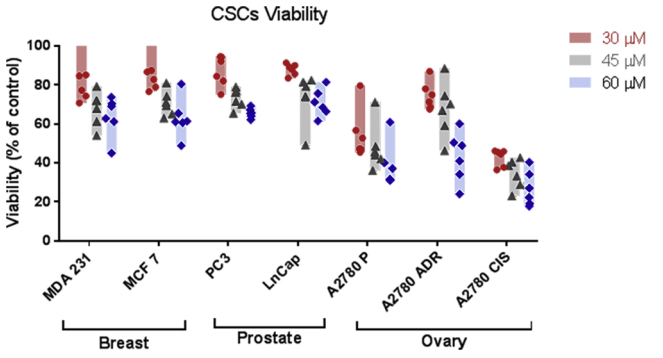


Fig. 1. Effect of 5-Azacytidine on viability: The CCK8 viability assay was performed after treatment of CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780P, A2780 ADR, and A2780 CIS) tumour cell lines with 5-Aza for 48 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison. Data are mean ± standard error of mean from 3 independent experiments.

2.2. DNMT1 inhibitor 5-Aza demethylates sFRPs' (1-5) promoter in vitro

To investigate direct association of *sFRP* promoter demethylation with increased *sFRP*-specific mRNA expression, we treated CSCs derived from all cell lines with 30 μM 5-Aza (Fig. 2). Using real time PCR, we determined the *sFRP* relative mRNA before and after drug treatment. The mRNA relative expression post-treatment indicated the *sFRP* promoter demethylation, showing elevated *sFRP* (*1-5*) expression in most of the tumour-specific CSCs. The *sFRP1* mRNA relative expression was significantly upregulated in all cell lines, suggesting demethylation post 5-Aza treatment, though A2780 Cis CSC was not statistically significant (Fig. 2A). The *sFRP2* mRNA relative expression was upregulated in MDA231 CSCs, A2780P CSCs, and A2780 Cis CSCs post treatment, but other CSCs did not undergo significant change (Fig. 2B). The *sFRP3* mRNA expressions were upregulated in 5 out of 7 CSCs derived from all cell lines, with the exception of MCF7 and A2780 Cis CSCs (Fig. 2C).

Demethylation of its promoter had a functional effect on relative expressions of *sFRP4* specific mRNA. Upon treatment with 5-Aza, expression levels were upregulated in 6 out of 7 tumour cell line-derived CSCs. The LnCap CSCs showed moderate upregulation of the promoter region post treatment, but this was not statistically significant (Fig. 2D). The *sFRP5* mRNA relative expression was significantly upregulated in 3 out of 7 tumour cell line-derived CSCs (Fig. 2E).

To further confirm the *sFRP* promoter region demethylation in CSCs of all originally methylated cell lines, the CSCs were treated with 5-Aza (30 μM for 48 h) to induce demethylation of their genomic DNA (gDNA). Isolated gDNA was bisulfite treated to deaminate unmethylated cytosine to produce uracil in gDNA, allowing us to detect unmethylated cytosines and 5-methylcytosines using methylation specific PCR (MSP). We used the highly sensitive MSP to detect methylation patterns in *sFRP* (*1-5*) promoter regions to distinguish between methylated and unmethylated DNA from CSCs derived from all cells lines (Fig. 3).

CSCs derived from all tumour cell lines exhibited a hypermethylated (M) promoter region in all *sFRPs* (*1-5*) in untreated cells, which confirmed the status of the methylated cell lines, except A2780 Cis in *sFRP1* (Fig. 3A) and *sFRP3* (Fig. 3C). Post 5-Aza treatment, most of the CSCs lacked the promoter methylation in analysed regions, indicating demethylation, except LnCap CSC in *sFRP4* (Fig. 3D) and breast CSCs (MDA231/ MCF7 CSCs) in *sFRP5* (Fig. 3E). The CSCs gaining an unmethylated promoter sequence after treatment consistently demonstrated upregulation of *sFRP* mRNA expression.

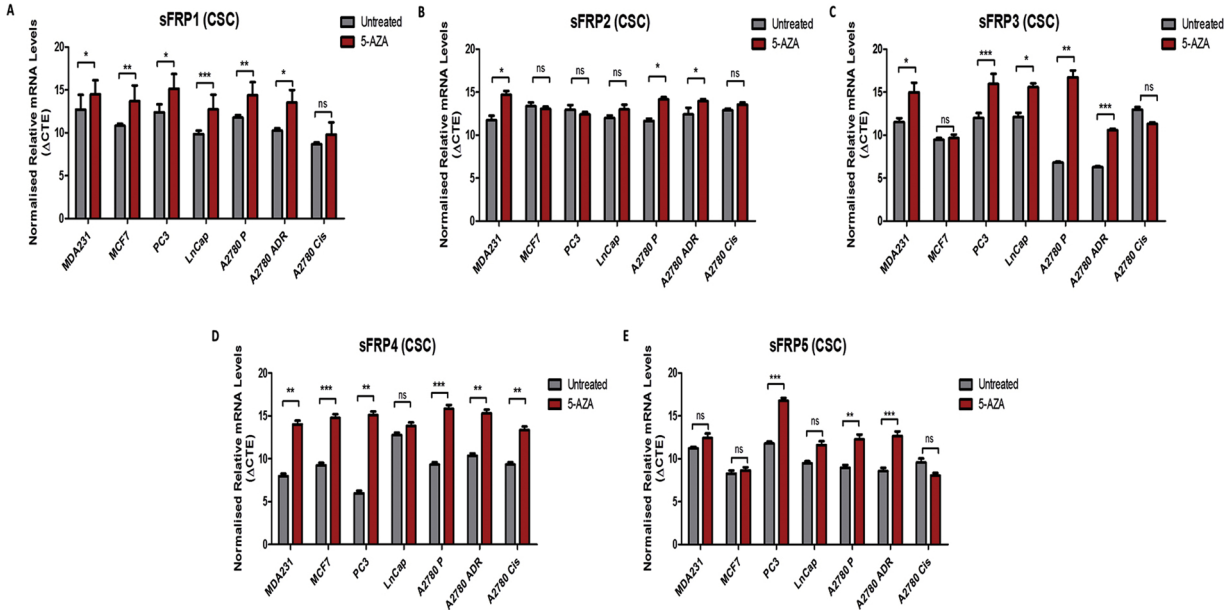


Fig. 2. Effect of *DNMT1* inhibition on gene expression: Real-Time quantitative PCR results for sFRP1, 2, 3, 4, and 5 analysed in CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative mRNA expression of *sFRPs (1-5)* following treatment of CSCs with 30 μM 5-Aza for 48 h compared to their untreated controls. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Data are mean ± standard error of mean from 3 independent experiments.

2.3. Demethylation regulates protein levels of Wnt signalling-associated molecules

The loss of methylation in all *sFRP (1-5)* promoter regions and increased mRNA expression post 5-Aza treatment in all CSCs prompted us to investigate the effect of *DNMT1* inhibition on protein levels of the Wnt antagonist sFRP4 and other key downstream effectors of Wnt signalling such as GSK3β, active β-catenin, and phospho β-catenin. The Western blot results demonstrated the effects of 5-Aza against untreated control (Fig. 4).

Following 5-Aza treatment, sFRP4 protein expression was significantly increased in all CSCs compared to the untreated CSCs, except in A2780 Cis CSCs (Fig. 4C). The levels of GSK3β were increased in 4 out 7 CSC cell lines; MCF7 CSCs had more than 50% elevation in expression compared to untreated CSCs; A2780 CSCs showed elevation in GSK3β protein levels but this was not statistically significant (Fig. 4C).

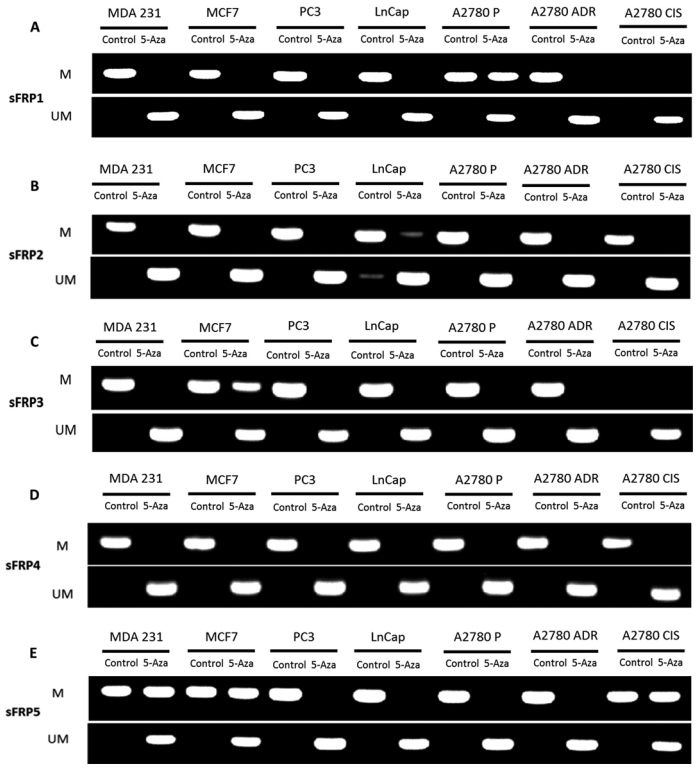


Fig. 3. Effect of *DNMT1* inhibition on methylation: Methylation specific PCR (MSP) results for sFRP1, 2, 3, 4, and 5 analysed in CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780P, A2780 ADR, and A2780 CIS) tumour cell lines. M: primer specific for methylated DNA. U: primer specific for unmethylated DNA. Semi-quantitative PCR images are representative of 3 independent experiments.

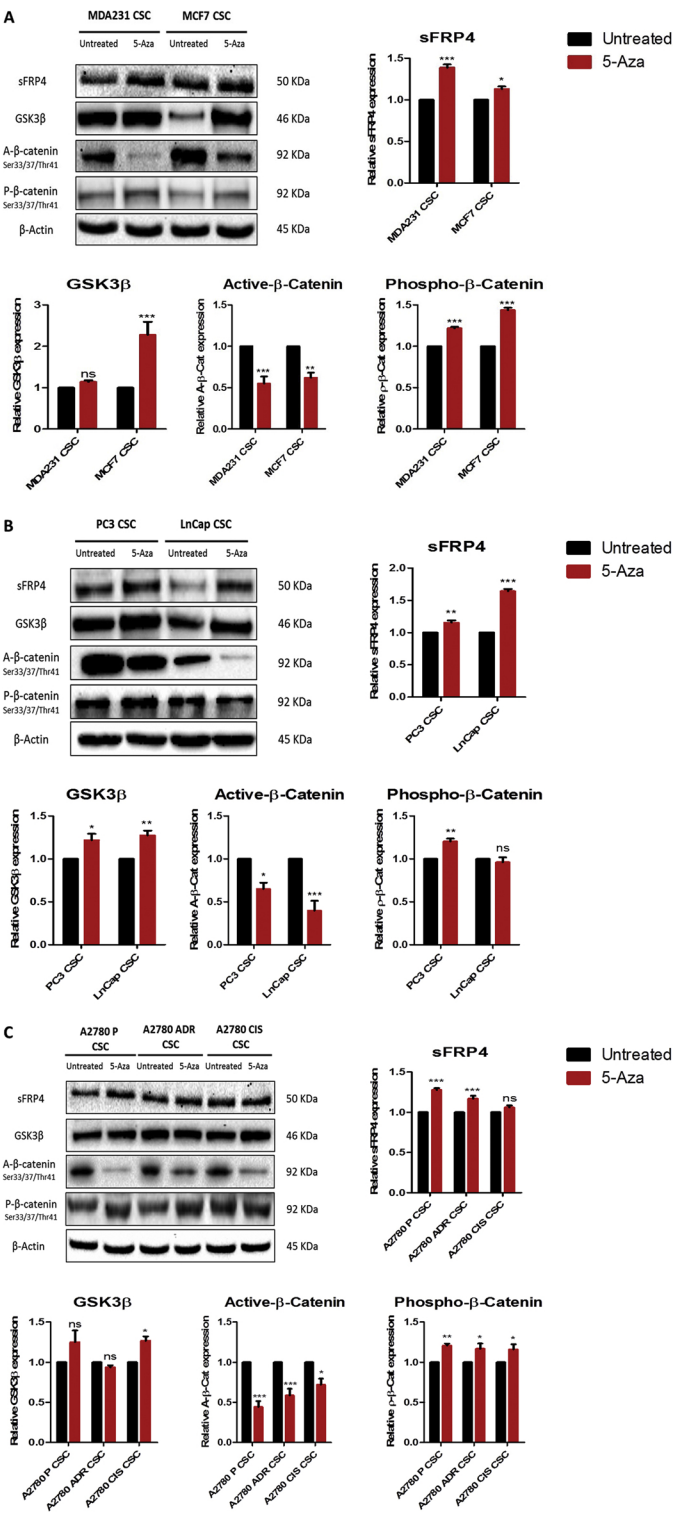


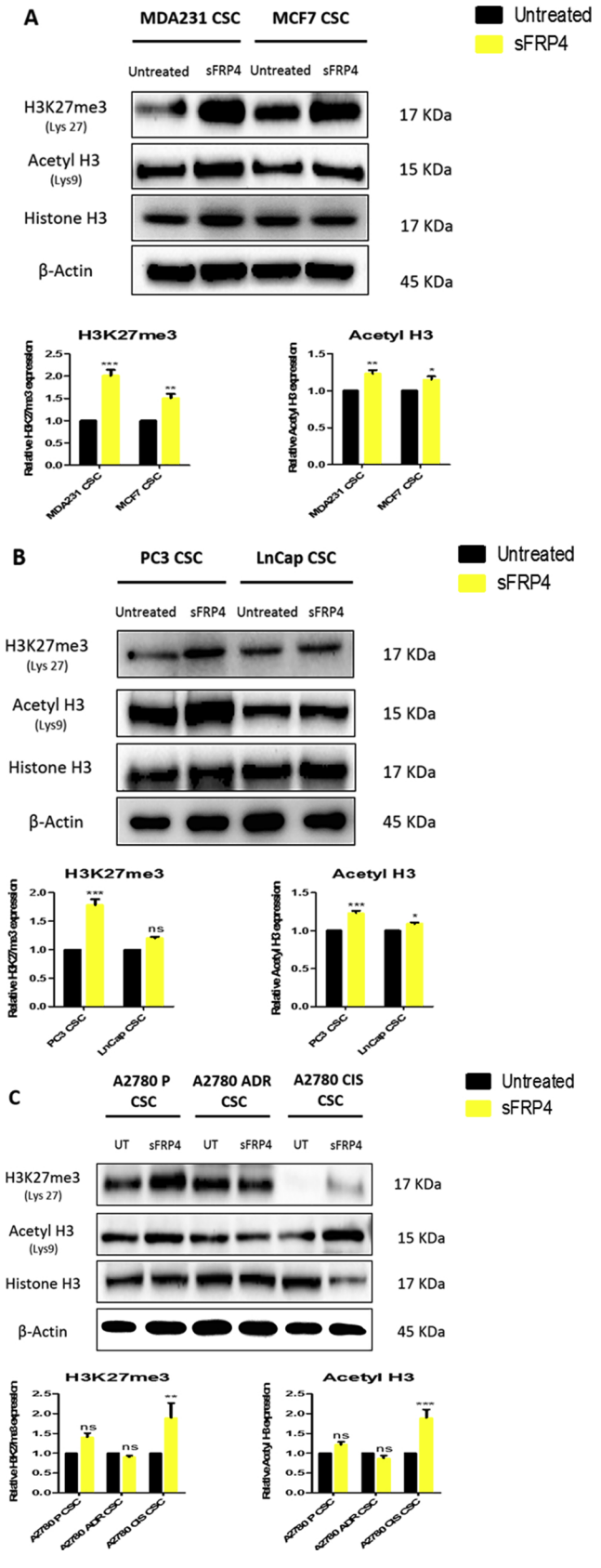
Fig. 4. Effect of 5-Azacytidine on protein expression levels: Western blot results for sFRP4, glycogen synthase kinase 3 beta (GSK3β), active β-catenin, and phosphorylated β-catenin analysed in CSCs derived from A) breast (MDA231 and MCF7), B) prostate (PC3 and LnCap), and C) ovary (A2780P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative protein expression of Wnt associated molecules in CSCs when treated with 30 μM 5-Aza for 48 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Blots and relative protein expressions are mean ± standard error of mean from 3 independent experiments.

Active β-catenin protein levels were significantly decreased in all CSCs compared to untreated CSCs, indicating the role of increased sFRP4 expression in deregulating Wnt activation. Phosphorylated β-catenin protein levels showed significant elevation in all CSCs, except LnCap CSCs (Fig. 4B). The levels of downstream effectors in CSCs indicate that post 5-Aza treatment, increased sFRP4 levels enable GSK3β to phosphorylate β-catenin at ser33/37/Thr41, hence decreasing the level of active β-catenin.

2.4. sFRP4 regulates histone modifications

The increased levels of sFRP4 post 5-Aza treatment in CSCs prompted us to investigate the post-translational effect of sFRP4 on histone modification and its epigenetic co-factors such as acetylation and methylation of histone H3. The Western blot results demonstrate the effects of sFRP4 against untreated control in CSCs (Fig. 5).

Post sFRP4 treatment, CSCs exhibited increased levels of tri-methyl histone H3 (H3K27me3) in 4 out of 7 CSC cell lines, whereas LnCap CSCs (Fig. 5B) and A2780P CSCs (Fig. 5C) demonstrated moderate



increased levels, but these were not statistically significant. Acetyl histone H3 protein levels were increased in breast (MDA231/ MCF7) and prostate (PC3/ LnCap) CSCs.

Fig. 5. Effect of sFRP4 on histone modifications: Western blot results for Tri-Methyl-Histone H3 (Lys27) H3K27me3, Acetyl-Histone H3 (Lys9) H3K9ac, and total histone H3 in CSCs derived from A) breast (MDA231 and MCF7), B) prostate (PC3 and LnCap), and C) ovary (A2780P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative histone expression of 250 pg/ ml sFRP4 for 24 h treated CSCs to untreated CSCs. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Blots and relative protein expressions are mean ± standard error of mean from 3 independent experiments.

The histone modification factors in CSCs post sFRP4 treatment indicate an increase in H3K27me3 levels, thereby inducing transcriptional repression.

3. Discussion

During tumor growth, many cancer cells differentiate to initiate tumorigenesis, though CSCs evade the cell cycle and remain in their resting phase (G0 Phase) (Adams and Strasser, 2008; Schatton et al., 2008), preserving their self-renewal capacity acquired during transformation. Considering the importance of epigenetic alterations such as DNA methylation, histone modifications, and chromatin remodelling in stem cell maintenance, many DNA-methylating enzymes and histone modifiers are essential to maintaining the CSC functional state. The epigenetic silencing process such as DNA methylation of their gene promotor in CSCs has been previously reported (Liu et al., 2017). DNA methyltransferase 1 (*DNMT1*) regulates DNA methylation in human genes, which was demonstrated in a study involving a leukaemic murine model, where wild type mice with *DNMT1* overexpression developed leukaemia, whereas *DNMT1* knockout did not (Bestor and Ingram, 1983; Chedin, 2011; Liao et al., 2015). Loss of *DNMT1* expression can be associated with loss of genomic stability and viability of cellular function (Brown and Robertson, 2007; Jackson-Grusby et al., 2001), which implicates the hypomethylation of tumour suppressor genes. Epigenetic silencing of gene families has profound implications for understanding the mechanistic basis of carcinogenesis and CSC emergence. The cell-intrinsic mechanisms and functional characterizations responsible for maintaining cellular hierarchies within cancer are crucial in the epigenetic modulation of tumours and CSC progression.

The Wnt cell-cell signalling pathway tightly regulates self-renewal and differentiation (Takebe et al., 2015). The downstream effectors of the Wnt signalling pathway can influence the crucial mediators of epigenetic factors that translate extracellular signalling into differential CSC phenotypes. The activation of canonical Wnt signalling in cancer is due to aberrant gene activation mediated through β-catenin (Polakis, 2012). The sFRP genes (*sFRP1, 2, 3, 4*, and *5*), which are Wnt antagonists, are silenced due to promoter hypermethylation in human carcinogenesis (Lee et al., 2004; Marsit et al., 2005; Schiefer et al., 2014; Suzuki et al., 2004). *SFRP1* and *sFRP2* have been found to be epigenetically silenced in human brain cancer such as astrocytic gliomas (Gotze et al., 2010), and *sFRP1* loss of expression has been attributed to promoter hypermethylation and allelic loss in papillary bladder cancer (Stoehr et al., 2004). Furthermore, *sFRP (1-5)* methylation was detected in liver cancer cell lines and primary hepatocellular carcinoma cells, indicating the methylation of these genes is an early event in liver carcinogenesis (Takagi et al., 2008). In addition, aberrant CpG island methylation was found in *sFRP1, 2, 4*, and *5* in acute myeloid leukaemia cells (Jost et al., 2008). Methylation of *sFRP1, 2*, and *5* was frequently detected in oral squamous cell carcinoma (OSCC), resulting in decrease of OSCC cell proliferation (Sogabe et al., 2008). In breast cancer cell lines, frequent methylation and silencing of *sFRP1, 2, 4*, and *5* has been observed, indicating the loss of sFRP functions contributes to activation of Wnt signalling in breast carcinogenesis (Suzuki et al., 2008). In glioblastoma multiforme cell lines, all *sFRP* isoforms were epigenetically silenced and were demethylated using the DNMT inhibitor 5-

Azacytidine (Schiefer et al., 2014).

In order to gain further insights into epigenetic alterations in CSCs, we investigated the methylation status of all sFRP family genes in CSCs derived from various tumour cell lines. Our results demonstrated DNA demethylation using the *DNMT* inhibitor, 5-Aza, in all CSCs; furthermore demonstrating that sFRP4 demethylation in CSCs would decrease the protein levels of Wnt signalling-associated molecules. We also investigated the activation of sFRP4 in CSCs to study histone modifications, demonstrating a correlation between promoter hypermethylation and post-translational modifications in CSCs derived from all the cell lines. The CSCs were identified from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780P, A2780 ADR, and A2780 CIS) tumor cell lines based on their ability to form spheroids in serum free conditions, elevated expression of CSC surface markers, high expression of ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogenes (c-Myc), and the ability to escape cell death/apoptosis (Bcl-xl) (Deshmukh et al., 2017b). Hypermethylation of *sFRP1, 2, 3, 4*, and *5* was detected in all CSCs examined before demethylation of their gDNA through incubation with 30 μM 5-Azacytidine for 48 h.

The *sFRP1* promotor regions are hypermethylated in renal cell carcinoma, as evidenced by downregulation of mRNA levels compared to normal kidney tissues. The complete loss of sFRP1 at the protein level substantiated its methylation mediated silencing (Dahl et al., 2007). We observed a similar outcome post 5-Aza treatment; there is an increase in mRNA levels of *sFRP1* promoter regions, indicating CSC DNA demethylation in 6 out of 7 cell lines. In addition, we observed *sFRP* mRNA abundance before and after 5-Aza treatment of CSCs, revealing significant upregulation of *sFRP2* (3 out of 7 cell lines), *sFRP3* (5 out of 7 cell lines), *sFRP4* (6 out of 7 cell lines), and *sFRP5* (3 out of 7 cell lines). The mRNA levels post-treatment indicate that sFRPs are transcriptionally silenced by epigenetic processes in CSCs. Moreover, we showed loss of methylation within sFRPs' promotor regions after treatment with 5-Aza, and CSCs gaining an unmethylated promoter sequence after treatment consistently showed upregulation of *sFRP* mRNA expression. Therefore, the findings suggest that epigenetic processes mediated through methylation of 5`-CpG-islands in the promotor regions of sFRPs are involved in epigenetic alterations within CSCs derived from the tumour cell lines examined.

In human mesothelioma cell lines, sFRP4 protein levels were decreased for all the hypermethylated promoter regions, indicating the correlation of epigenetic modulations to post-translational modification in the Wnt signalling pathway (He et al., 2005; Zou et al., 2005). In another study, treating myeloid leukaemia cells with 5-Aza induced the expression of previously methylated *sFRP1, 2, 4*, and *5*, and resulted in inactivation of the Wnt pathway by downregulating important Wnt genes such as *cyclin D1, TCF1*, and *LEF1* (Valencia et al., 2009). Our Western blot analyses demonstrated increased sFRP4 expression, GSK3β expression, and phosphorylated β-catenin expression after demethylating treatment, which confirms our hypothesis that methylation appears to functionally silence sFRP4 gene expression in CSCs. To further validate the relation between demethylation and inactivation of Wnt signalling, we analysed the important Wnt pathway-associated molecules, demonstrating a decrease in protein levels of active (unphosphorylated) β-catenin.

Post-translational modifications (PTM) of N-terminal tails of histones, which includes methylation, acetylation, ubiquitination, and phosphorylation, can modulate transcription factors and transcriptional activity of genes (Skinner, 2014). The regulation of genetic materials in cancer is controlled by histone modifications and is suspected to be involved in tumour progression. In our study, we further investigated the role of sFRP4 in histone H3 modifications such as methylation and acetylation.

The polycomb repressive complex 2 (PRC2) and DNA methylation in CSCs can suppress their differentiation capacity (Momparler and Cote, 2015). PRC2 consists of enhancer zeste homolog 2 (EZH2), which acts as a catalyst for tri-methylation of histone 3 lysine 27 (Momparler and Cote, 2015). EZH2 is a critical enzymatic component of polycomb repressive complex 2, which methylates histone H3 on dimethyl lysine-27 to generate H3K27-trimethyl and silences gene transcription (Sellers and Loda, 2002). EZH2 is best known to function as the H3K27 methyltransferase within PRC2 in polycomb-mediated silencing. Interestingly, recent studies have highlighted some non-canonical roles of EZH2, indicating that EZH2 can act as a transcriptional activator instead. In addition, EZH2 can function independently of PRC2 and, in some cases, independently of its HMT activity. In CSCs, the chromatins recruit DNMTs, leading to *ex novo* methylation and gene silencing, and leading to silencing of tumour suppressor genes during tumour progression; however, H3K27 can also silence tumour genes without DNMT recruitment (Kondo et al., 2008). The polycomb gene complex (PcG) target genes are more likely to be hypermethylated in colon and embryonic carcinoma cell lines (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007), indicating that EZH2 recruits DNMT to promoter regions of PcG target regions and silences tumour suppressor genes (Vire et al., 2006). These studies establish the crosstalk between DNA methylation and histone modifications, which result in a repressive effect. The sFRP4 treatment led to increased methylation of the H3K27me3 target, though there is increasing evidence of overexpression of EZH2 leads to an increase in global H3K27me3 levels and a decrease in mono- and dimethylation at this residue, but none of these observations are in CSCs. EZH2 plays an important role in CSCs, including repression of tumour suppressor genes (Bitler et al., 2015; Chang et al., 2011; Suva et al., 2009; Varambally et al., 2002), and β-catenin stabilization (Zhu et al., 2016). As a consequence of the cellular changes induced by transcription and epigenetic alterations, the CSCs become dependent on EZH2 to preserve their self-renewal capacity (Suva et al., 2009). SFRP4 is a known H3K27me3 marked gene that is not regulated by differentiation. Reduction of HeK27Me3 marks at a subset of epidermal differentiation genes during differentiation suggests potential for reciprocal action by PcG proteins and histone demethylases in this process. In most CSCs, H3K27Me3 shows an increased association with sFRP4 (Schlesinger et al., 2007). Due to a high degree of association with the H3H27Me3 domains, it was shown that sFRP4 alleles are preferentially located at the nuclear or perinucleolar regions (Easwaran et al., 2010). SFRP4 exhibits a preference for the H3K4Me2-labelled euchromatin and is excluded from the H3K4Me-2-marked heterochromatin.

We also investigated the effect of sFRP4 activation on Histone H3 acetylation, as it is an important modification related to DNA repair, cell cycle regulation, and transcription activation (Debeb et al., 2016). Addition of an acetyl group in transcription causes DNA denaturation, which is a hallmark for cancer cell therapy. Deficiency of Histone H3 acetylation has been detected in tumour progression of prostate cancer patients (Cang et al., 2009), and hyper-acetylation of H3K56 has been reported in hepatocellular carcinoma (Bai et al., 2008). The naturally occurring compound curcumin induced histone H3 and H4 acetylation, leading to apoptotic cell death in brain cancer (Kang et al., 2006). In our study, we observed that pro-apoptotic sFRP4 led to acetylation in all CSCs, affecting their self-renewal capacity and tumour progression, which is a feature of sFRP4's action (Bhuvanalakshmi et al., 2015; Deshmukh et al., 2017a, b). This is in agreement with the findings of Meyer et al., who showed that increased acetylation leads to a decrease in DNA damaged-induced activation of ataxia telangiectasia mutated kinase and DNA repair efficiency in stem cells (Meyer et al., 2016).

4. Materials and methods

4.1. Cell culture

4.1.1. Monolayer cell culture

Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast tumour cell lines MDA-MB

231 (ER-) and MCF-7 (ER+), human ovarian tumour cell lines A2780P, A2780-ADR, and A2780-Cis, and human prostate tumour cell lines PC-3 (AR-/PSA-) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco #11875-093) supplemented with 10% foetal bovine serum (Bovogen #SFBS) and 100 U/ml PenStrep (Life Technologies #15070063). All cells were maintained at 37 °C in a humid incubator with 5% CO_2.

4.1.2. Cancer stem cell isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing basal medium RPMI-1640 + DMEM-HG (HyClone, USA #SH30081.02) supplemented with the growth factors bFGF (20 ng/ml) (ProSpec Bio #cyt-085), EGF (20 ng/ml) (ProSpec Bio #cyt-217), and 1 × B27 (Gibco #17504044), and 100 U/ml PenStrep (Life Technologies #15070063). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast, ovary, and prostate tumor cells at 10,000 cells/cm^2 in SFM on Low-adherent six-well plates (Corning #3471). CSCs were isolated in SFM; the spheroids are formed at the 3rd day of plating tumour cells. To analyse the effects of 5-Azacytidine (5-Aza) and sFRP4, cells were cultured in medium supplemented with the compounds.

4.2. Drug treatment

The drugs used in this study were 5-Azacytidine (Sigma-Aldrich #A2385) and purified sFRP4 (R&D Systems #1827-SF-025). CSCs were incubated with 30 µM 5-Aza for 48 h after a 48 h seeding period since this dose and treatment time provided consistent reduction in cell viability across all cell lines. The dose range of 5-Aza was narrowed based on our previous publication (Schiefer et al., 2014). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/ml (Deshmukh et al., 2017b) for 24 h at 37 °C in a 5% CO_2 incubator.

4.3. Viability assay

A cell counting viability kit (CCK8, Sigma- Aldrich #96992) was used for the quantitation of viable cells. Monolayer cells were plated at a density of 10,000 cells/cm^2 in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. Wells with drug-free medium were used as a negative control. CSCs were treated with 5-Aza for 48 h, then 10 µl of CCK8 solution was added to each well and incubated at 37 °C in a 5% CO_2 incubator for 1 h. Plates were read at 450 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

4.4. Cell surface markers

To assist in determining the cells' identity, the expression of cell surface markers was examined in CSCs by flow cytometry (BD FACS-CANTO II), as previously published (Deshmukh et al., 2017b).

4.5. Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using TRIzol reagent (Life Technologies #15596026) followed by chloroform extraction, isopropanol precipitation, and a 75% (v/v) ethanol wash. RNA samples (1 µg) were reverse-transcribed to cDNA using a High Capacity cDNA kit (Applied Biosystems #4368814). cDNA in 1 µl of the reaction mixture was amplified with PCR Master Mix (Life Technologies #K0171) and 10 µmol each of the sense and antisense primers. Quantitative real-time (qPCR) was performed using a CFX Connect Real time System (Bio-Rad). Reaction mixtures consisted of 5 µl of 2 × KAPA SYBR Fast (Sigma-Aldrich #KK4601), 0.5 µM of each forward and reverse primer, 1 µl of template, 0.2 µM ROX low, and RT-PCR Grade water (Life Technologies #AM9935) adjusted to 10 µl. Each reaction mixture was loaded into 96-well PCR plates and amplified under PCR cycling conditions against 2 housekeeping genes (*18S* and *GAPDH*), followed by a melt-curve analysis. Each cycle consisted of denaturation at 95 °C for 10 s, annealing at 65 °C for 10 s and extension at 72 °C for 15 s and these conditions were maintained constant across 40 cycles. The primer sequences are described in Table 1.

Table 1
qPCR Primer Sequences and annealing temperatures.

Gene	Primer	Base Pair (bp)	Annealing Temperature (°C)
sFRP1	F – 5' atctctgtgccagcgagttt 3' R – 5' aagtggtggctgaggttgtc 3'	202	65
sFRP2	F – 5' aggacaacgacctttgcatc 3' R – 5' ttgctcttggtctccaggat 3'	217	65
sFRP3	F – 5' aaactgtagaggggcaagca 3' R – 5' ggcagccagagctggtatag 3'	227	65
sFRP4	F – 5' cgatcggtgcaagtgtaaaa 3' R – 5' gacttgagttcgagggatgg 3'	181	65
sFRP5	F – 5' gatgtgctccagtgactttg 3' R – 5` gcaggggtaggagaacatga 3'	322	65
18S	F – 5' gcaattattccccatgaacg 3' R – 5' gggacttaatcaacgcaagc 3'	68	65
GAPDH	F – 5' cagaacatcatccctgcatccact 3' R – 5' gttgctgttgaagtcacaggagac 3'	258	65

4.6. Genomic DNA isolation

Genomic DNA was extracted from all cell lines using a QIAamp DNA Mini Kit (Qiagen #51304) according to the manufacturer's instructions. DNA integrity and quality were evaluated by spectrophotometric analysis.

4.7. Bisulfite treatment

Briefly, 1 µg of genomic DNA was bisulfite treated with the EpiTect Bisulfite Kit (Qiagen #59104) according to manufacturer's instructions. The methylation status of genomic DNA was analysed using methylation-specific PCR.

4.8. Methylation-specific PCR

The methylation status of all 5 sFRP genes in the human cell lines was determined by methylation-specific polymerase chain reaction (MSP). Briefly, 1 µg of genomic DNA was bisulfite-treated with the EpiTect Bisulfite Kit (Qiagen #59104). The bisulphite conversion thermal cycler conditions were in following order, denaturation at 95 °C for 5 min, incubation at 60 °C for 25 min, denaturation at 95 °C for 5 min, incubation at 60 °C for 85 min, denaturation at 95 °C for 5 min, incubation at 60 °C for 175 min, rest at 20 °C. Bisulfite-treated DNA was amplified using 10 µl of 2x PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µM of each forward and reverse primer, 1 µl of template DNA, and RT-PCR Grade water (Life Technologies #AM9935) adjusted to a final volume of 20 µl. Each reaction was loaded in PCR tubes and amplified in following thermal cycler conditions, denaturation at 95 °C for 30 s, annealing at 60–68 °C, extension at 72 °C for 30 s and these conditions were maintained constant for 35 cycles. PCR products were analysed by agarose gel electrophoresis. Previously described primers specific for either methylated or unmethylated DNA are shown in Table 2.

4.9. Western blotting

Cells were washed twice with PBS and then lysed in RIPA lysis

Table 2
Methylation-Specific PCR Primer Sequences and annealing temperature.

Gene	Specificity	Primers	Annealing Temperature (°C)
sFRP1	Methylated	F – 5' gtgtcgcgcgttcgtcgtttcgc 3' R – 5' aacgttacccgactccgcgaccg 3'	60
	Unmethylated	F – 5' gagttagtgttgtgtgtttgttgttttgt 3' R – 5' cccaacattacccaactccacaacca 3'	60
sFRP2	Methylated	F – 5' gggtcggagtttttcggagtcgcgc 3' R – 5' ccgctctcttcgctaaatacgactcg 3'	62
	Unmethylated	F – 5' ttttgggttggagtttttggagttgtgt 3' R – 5' aacccactctcttcactaaatacaactca 3'	66
sFRP3	Methylated	F – 5' ggagcgggtttttggcg 3' R – 5' gaaccccgaacacccgaaa 3'	65
	Unmethylated	F – 5' ggagtgggtttttggtgtttattgt 3' R – 5' cccaaaccccaaacaccca	63
sFRP4	Methylated	F – 5' gggtgatgttatcgtttttgtatcgac 3' R – 5' cctcccctaacgtaaactcgaaacg 3'	63
	Unmethylated	F – 5' gggggtgatgttattgtttttgtattgat 3' R – 5' cacctcccctaacataaactcaaaaca 3'	60
sFRP5	Methylated	F – 5' aagatttggcgttgggcgggacgttc 3' R – 5' actccaacccgaacctcgccgtacg 3'	65
	Unmethylated	F – 5' gtaagatttggtgttgggtgggatgttt 3' R – 5' aaaactccaacccaaacctcaccataca 3'	68

buffer (Sigma #R0278) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Proteinase Inhibitor 1 ×). Post sonication, cell lysates were centrifuged at 14,000*g* for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma #P3504) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies sFRP4 (1 µg/ml, Abcam #ab32784); GSK3β (27C10) (1:1000, Cell Signaling #9315); Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (D13A1) (1:1000, Cell Signaling #8814); Phospho-β-catenin (Ser33/37/Thr41) (1:1000, Cell Signaling #9561); Histone H3 (D1H2) (1:2000, Cell Signaling #4499); Acetyl-Histone H3 (Lys9) (C5B11) (1:1000, Cell Signaling #9649); Tri-Methyl-Histone H3 (Lys27) (C36B11) (1:1000, Cell Signaling #9733), and β-Actin (13E5) (1:1000, Cell Signaling #4970) overnight at 4 °C, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE #RPN2106) on a Chemi-Doc (Bio-Rad) imaging analyser.

To study the effect of sFRP4 on post-translational modification in histones, CSCs were treated with sodium butyrate (Sigma-Aldrich #B5887) to inhibit histone deacetylase (HDAC) and retain acetylation levels.

4.10. Statistics

Densitometry analysis of western blots done using Image Lab 4.1 version. Statistical analysis was performed with GraphPad Prism V5.0 (GraphPad software, La Jolla, USA) using two-way ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Data are presented as mean ± standard error of mean.

5. Conclusions

Considering the epigenetic diversity seen in cancers, modulations among CSCs are still under scrutiny. The prospect of epigenetic alterations in CSCs is important in cancer research as it has led to the approval of clinical drugs inhibiting epigenetic modifiers, such as histone deacetylases (HDAC) and methyltransferases (HMT) (Cherblanc et al., 2012; Connolly and Stearns, 2012; Hatzimichael and Crook, 2013; Heyn and Esteller, 2012; Ho et al., 2013; Kasinski and Slack, 2011; Tinari et al., 2012; Verbrugge et al., 2011). In summary, our data suggest that epigenetic silencing of *sFRPs* in CSCs derived from breast, prostate, and ovary tumour cell lines is most likely due to hypermethylation of their promoter regions. Activation of *sFRP4* by inhibiting DNA methyltransferase indicated the influence on downstream effectors of Wnt-associated molecules. Taking into account the pro-apoptotic and chemo-sensitization capability of sFRP4, sFRP4-led epigenetic modulations and histone modifications in CSCs might serve as a potential therapeutic approach towards CSCs.

Author contributions

AbhiD drafted the outline and generated the data. AbhiD wrote the manuscript. AbhiD and ArunD conceived the study, and ArunD, FA, and PN critically reviewed, revised, and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest,

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